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Characterization of a Cell Surface Protein of *Clostridium difficile* with Adhesive Properties

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Our laboratory has previously shown that *Clostridium difficile* adherence to cultured cells is enhanced after heat shock at 60°C and that it is mediated by a proteinaceous surface component. The present study was undertaken to identify the surface molecules of this bacterium that could play a role in its adherence to the intestine. The *cwp66* gene, encoding a cell surface-associated protein of *C. difficile* 79-685, was isolated by immunoscreening of a *C. difficile* gene library with polyclonal antibodies against *C. difficile* heated at 60°C. The Cwp66 protein (66 kDa) contains two domains, each carrying three imperfect repeats and one presenting homologies to the autolysin CwlB of *Bacillus subtilis*. A survey of 36 strains of *C. difficile* representing 11 serogroups showed that the 3′ portion of the *cwp66* gene is variable; this was confirmed by sequencing of *cwp66* from another strain, C-253. Two recombinant protein fragments corresponding to the two domains of Cwp66 were expressed in fusion with glutathione *S*-transferase in *Escherichia coli* and purified by affinity chromatography using gluthatione-Sepharose 4B. Antibodies raised against the two domains recognized Cwp66 in bacterial surface extracts. By immunoelectron microscopy, the C-terminal domain was found to be cell surface exposed. When used as inhibitors in cell binding studies, the antibodies and protein fragments partially inhibited adherence of *C. difficile* to cultured cells, confirming that Cwp66 is an adhesin, the first to be identified in clostridia.

Clostridium difficile, a gram-positive spore-forming anaerobic bacterium, is an important nosocomial enteric pathogen, causing pseudomembranous colitis and many cases of antibiotic-associated diarrhea (10). Various established and putative virulence or colonization factors have been described. Pathogenesis is mainly due to two toxins, toxins A and B (26). Other factors might be involved in colonization, such as (i) the capsule, which may confer protection against phagocytosis (6); (ii) production of tissue degradative proteases, e.g., collagenase and hyaluronidase, which may play a role in releasing suitable substrates from available protein sources for metabolism (17, 18, 29, 30); and (iii) flagella (31).

Concerning adherence as a colonization factor, it is clearly established that *C. difficile* can associate with intestinal mucosa in humans (1) and hamsters (3). There appears to be a positive correlation between virulence and mucosal adherence in vivo (3). *C. difficile* has been shown to adhere to a variety of cultured cell lines including Caco2, HT29-MTX, and Vero cells and adherence is mediated by proteinaceous components (8, 16, 34). In addition, *C. difficile* has been shown to produce fimbriae, which are potential mediators of adherence, although their role has not been demonstrated yet (2). Like many other bacteria, *C. difficile* might have multiple adhesins.

We have focused our studies on identifying surface proteins of *C. difficile* that could play a role in the adherence to and colonization of the intestine. In this report we describe the cloning and characterization of the *cwp66* gene, encoding a *C. difficile* surface protein with repeated motifs and homology to the *cwlB* autolysin gene of *B. subtilis*. Thirty-six *C. difficile* isolates representing 11 serogroups were screened for the presence of and variability in the *cwp66* gene. The role of Cwp66 in binding to cells was also investigated.

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MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The *C. difficile* isolates used are presented in Table 1. They were grown anaerobically (85% N_2, 10% H_2, 5% CO_2) in TGY (tryptone-glucose-yeast extract broth) (Difco). The λZap Express cloning system, the SuperCos 1 cosmid vector, the pBC vector, and *Escherichia coli* strains XL1-BlueMRF′ and XLOLR were purchased from Stratagene. *E. coli* DH5αMCR was purchased from Life Technologies. The pGEX-6P1 expression vector and recipient strain *E. coli* BL21 were obtained from Pharmacia-Biotech. *E. coli* strains were grown in Luria-Bertani broth, Luria-Bertani agar (1.5%), or 2x-YT broth (28). Ampicillin (100 μg/ml), carbenicillin (60 μg/ml), kanamycin (50 μg/ml), or chloramphenicol (50 μg/ml) was added to broth or agar plates when needed.

DNA manipulations, PCR, and sequencing. Plasmid and cosmid isolations were performed by the alkali lysis procedure using a kit from Qiagen. Ligations and restriction endonuclease digestions were done by the method of Sambrook et al. (28) and using protocols provided by vendors. The transformation and storage buffer (TSB) method was used for transformation of *E. coli* (5). Genomic DNAs from *C. difficile* strains were isolated with the Puregene genomic DNA isolation kit (Prolabo).

PCR was performed to generate fragments of the cloned DNA with Promega *Taq* DNA polymerase (1 U/100-μl reaction volume), 4 mM $MgCl_2$, 200 pM each deoxynucleoside triphosphate, and 1 μM each primer for 30 cycles consisting of denaturation at 92°C (1 min), annealing at 52°C (1 min), and extension at 72°C (2 min) in a Perkin-Elmer Thermocycler 480. The primers used (Life Technologies) are shown in Table 2.

Automatic DNA sequencing was performed with the BigDye terminator cycle-sequencing kit (Perkin-Elmer) and analyzed with an ABI PRISM 310 genetic analyzer (Perkin-Elmer).

TABLE 1. Strains of *C. difficile* used in this study

Strain	Serogroup	Toxins present	Origin
79685	S3	+	I. Scheffel, Institut de Bactériologie, Strasbourg, France
VPI 10463	G	+	T. D. Wilkins, Virginia Polytechnic Institute and State University, Blacksburg, Va.
W1194	A	+	M. Delmée, Université Catholique de Louvain, Louvain, Belgium
1351	B	−	
545	C	+	
3232	D	−	
1470	F	A− B+	
2022	G	+	
2149	H	+	
7322	I	−	
4811	K	−	
5036	X	−	
M1	ND[a]	−	P. Borriello, PHLS Central Public Health Laboratory, London, United Kingdom
Kohn	A	−	
93-54	A	+	A. Collignon, Hôpital J. Verdier, Bondy, France
93-369	H	+	
90-204	H	+	
89-638	H	+	
90-111	D	−	
93-136	D	−	
93-226	D	−	
93-296	D	−	
93-379	D	−	
C-253	C	+	P. Mastrantonio, Istituto Superiore Di Sanità, Rome, Italy
94-416	K	+	F. Barbut, Hôpital St-Antoine, Paris, France
94-1155	K	+	
96-631	K	+	
95-938	G	+	
96-392	G	+	
96-1827	G	+	
96-1348	G	+	
95-1078	C	+	
96-602	C	+	
94-1456	C	+	
96-1578	C	+	
630	ND	+	P. Mullany, Eastman Dental Institute, London, United Kingdom

[a] ND, not determined.

Southern and dot blot hybrizations. For Southern blotting, 2 μg of genomic DNA of *C. difficile* 79-685 was digested overnight at 37°C with *Hin*dIII, *Dra*I, *Alu*I, *Acc*I, and *Eco*RV (1 U/μg of DNA, Life Technologies), electrophoresed in a 0.8% agarose gel, and electrically transferred to a nylon membrane (Roche). For dot blotting, 10 μg of DNA of *C. difficile* strains was alkali-transferred (28) onto a nylon membrane with a Minifold I dot blotter (Schleicher & Schuell). Membranes were then baked 20 min at 120°C and probed with PCR-amplified labeled DNA fragments (Table 2), which were labeled with peroxidase and detected by the ECL direct nucleic acid-labeling and detection system from Amersham-Pharmacia Biotech as specified by the manufacturer. Hybridizations were performed overnight at 42°C, and high-stringency washes were performed before detection with the ECL chemiluminescent substrate.

Construction and screening of *C. difficile* libraries. (i) Phage library in λZap II. A genomic library of strain 79-685 was previously constructed in λZap II (Stratagene) in our laboratory (16). Approximately 50,000 PFU was screened, as described by Karjalainen et al. (16), with a 1/1,000 dilution of adsorbed rabbit antibodies raised against heat-shocked *C. difficile* (see below).

(ii) Cosmid library. To obtain the complete sequence of *orfB* (the *cwp66* gene), a cosmid library of *C. difficile* 79-685 was constructed from *Sau*3A (Life Technologies)-digested genomic DNA of strain 79-685 in SuperCos1 vector (Stratagene). Approximately 1,000 colonies of the library, having a titer of 4×10^3 CFU/ml and insert sizes ranged from 8 to 25 kb, were screened by colony hybridization using probe D (Table 2; see Fig. 1A) as specified in the ECL detection kit. Inserts of three positive clones were digested with *Eco*RI and cloned into the pBC vector. Sequencing of one subclone allowed us to obtain a additional 900 bp of *cwp66*, which, however, still lacked the stop codon.

(iii) Phage library in λZap Express. The end of *orfB* was obtained by constructing and screening a third phage library constructed in λZAP Express (Stratagene) from partially digested genomic DNA of strain 79-685 with *Hin*dIII (Life Technologies), using protocols provided in the Gigapack III Gold packaging kit (Stratagene) and the λZap Express vector kit. Approximately 30,000 PFU of the library, with a titer of 4×10^7 PFU/ml, was plated on petri dishes, transferred onto a nylon membrane, and screened with probe E (Table 2; see Fig. 1A).

(iv) Phage library of strain C-253. The library was a gift from P. Mastrantonio, Istituto Superiore Di Sanità, Rome, Italy. The library was screened by plaque hybridization with probe F (Table 2; see Fig. 1A).

Cloning into the expression vector pGEX-6P-1. To clone the *cwp66* gene into pGEX-6P-1, two sets of oligonucleotide primers, *cwp66*-N1 plus *cwp66*-Crev and *cwp66*-N2 plus *cwp66*-Crev (Table 2), each incorporating a *Bam*HI restriction site at the 5′ end and a *Sal*I restriction site at the 3′ end of the gene, were used to amplify by PCR the full-length coding region of the *cwp66* gene with and without the region encoding the peptide signal, respectively. The resulting DNA product was digested with *Bam*HI and *Sal*I and cloned into the corresponding sites of pGEX-6P-1 in frame with the glutathione *S*-transferase (GST) gene (*gst*) The same protocol was used to clone (i) the first half of the gene, from nucleotides 104 to 941 of *cwp66*, using primers *cwp66*-N2 and *cwp66*-Nrev, incorporating *Bam*HI and *Eco*RI restriction sites, respectively, and (ii) the second half of the gene, from nucleotides 929 to 1870 of *cwp66*, using primers *cwp66*-C and *cwp66*-Crev, incorporating *Eco*RI and *Sal*I restriction sites, respectively, into the corresponding sites of pGEX-6P-1. Nucleotide sequencing of the junctions between the vector and inserts confirmed that the gene or gene fragment were inserted in frame with *gst*. pGEX-6P-1 carrying portions of *cwp66* was transformed into *E. coli* BL21.

Expression and purification of recombinant proteins. To induce expression of the recombinant proteins, clones were grown in 4 liters of 2X-YT broth plus 2% glucose at 30°C to an optical density at 600 nm of 0.6. At this point, 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added and incubation was continued for 2 h at room temperature. The recombinant proteins were purified in accordance with the standard protocol provided by Amersham Pharmacia-Biotech.

Antibody production. (i) Anti-*C. difficile* sera. Antibodies were raised against heat-shocked *C. difficile* whole cells as described previously (16). The antibodies were adsorbed against non-heat-shocked *C. difficile* and against an *E. coli* lysate before being used in library screening (28).

(ii) Anti-Cwp66-N and Anti-Cwp66-C sera. Rabbit polyclonal, monospecific Cwp66-N and Cwp66-C antisera were prepared by cutting out of the polyacrylamide gel the band corresponding to the purified recombinant proteins, injecting lyophilized preparations (200 μg) in Freund's complete adjuvant into New Zealand White rabbits, and then administering three boosters with 100 μg of protein in Freund's incomplete adjuvant on days 14, 28, and 42. The rabbits were sacrified and bled 21 days after the last injection. Antibodies were purified on protein A-Sepharose (Amersham Pharmacia-Biotech) as recommended by the supplier and used at a 1/2,000 dilution in immunoblots.

(iii) Anti-FliC and anti-PepC sera. A rabbit polyclonal serum against *C. difficile* flagellin protein FliC was raised in our laboratory as described previously (31). Antibodies against the cytoplasmic peptidase of *Lactococcus lactis* PepC were a gift from M.-Y. Mistou, INRA, Jouy-en-Josas, France.

TABLE 2. Oligodeoxyribonucleotides used for amplification and probes

Primer or probe	Sequence or primers used for amplification	Position (bp)	Restriction site
cwp66-Arev	5′ CTG ATT CAG AGA ATG GAA CTG 3′	556–576	
cwp66-S	5′ CTC CCA CRG CTA AAG AAT CTA 3′	825–805	
cwp66-Mrev	5′ GGT ACT TTG ATT AAT GAA GGC 3′	1239–1259	
cwp66-T	5′ GAT ATT GTC ACT CTT ACA CG 3′	1594–1575	
cwp66-A2rev	5′ TGA CAA TTC CAT AGC AGA TGC 3′	179–200	
cwp66-N1	5′ GGA GGT AAG GAT CCA TGA AAA TAT C 3′	−14–11	*Bam*HI
cwp66-N2	5′ TTA ACG GGA TCC GGA AGA TGG G 3′	97–118	*Bam*HI
cwp66-Nrev	5′ TTC ATT ACC GAA TTC ACC AAT TTG AG 3′	951–926	*Eco*RI
cwp66-C	5′ AAG TTA CTG AAT TCG GTG GCT TAG G 3′	920–939	*Eco*RI
cwp66-Crev	5′ TAA AAA CTC GAC TAC TAG AAA TAG TAA TCT AC 3′	1877–1846	*Sal*I
Probe D	Arev and S	556–805	
Probe E	Mrev and T	1239–1575	
Probe F	A2rev and T	179–1575	

Fractionation and SDS-PAGE of *C. difficile* proteins. Bacterial proteins were separated into four compartments, supernatant, cell wall, membrane, and cytoplasm, using a method described for *Listeria monocytogenes* (15). Equivalent amounts of each fraction measured by the Bio-Rad *DC* protein assay kit (Bio-Rad Laboratories), corresponding to 20 ml of bacterial culture, were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% polyacrylamide) as described by Laemmli (21). The gels were stained with Coomassie blue or used for immunoblotting as described below. The purity of the fractions was verified by studying PepC, a cytoplasmic peptidase reported to be adsorbed to the inner face of the cytoplasmic membrane in *L. lactis* (15), as a marker for cytoplasmic and membrane fractions.

Immunoblotting. Proteins separated by SDS-PAGE were transferred electrically onto nitrocellulose membrane (Trans-Blot transfer medium; Bio-Rad Laboratories) by the method of Towbin et al. (32). The nitrocellulose membrane was incubated for 30 min at room temperature in blocking buffer (0.2% Tween and 3% skim milk in phosphate-buffered saline [PBS]) and then overnight in the appropriate dilution of the specific antibody. The membranes were screened for signal as described for the phage library in λZapII (16).

Immunoelectron microscopy. Cultures of *C. difficile* 79-685 (24 h old) were washed and resuspended in PBS. A single drop of the bacterial suspension was placed onto Formvar-coated nickel grids (Sigma) for 5 min, and excess moisture was removed by absorption. Then the grids were inverted onto drops (25 μl) of PBS plus 1% bovine serum albumin (BSA) for 30 min and incubated for 1 h with a 10-fold dilution of anti-Cwp66-C or anti-Cwp66-N antibodies. After three washes in PBS, they were incubated with a 1/20 dilution of 10-nm-diameter colloidal gold particle-labeled protein A (Sigma) for 1 h. The grids were subsequently washed as before, fixed with 3% glutaraldehyde, washed again three times, and stained with 0.5% phosphotungstic acid before being observed by transmission electron microscopy.

Cell culture, cell adherence, and adherence inhibition assays. Maintenance and preparation of the Vero cells, as well as cell adherence assays, were performed as previously described (16). The number of adherent bacteria per cell was counted by using a light microscope at a magnification of ×1,000. Adherence index is given as means and standard deviation from at least three different assays.

To screen *E. coli* clones for adherent properties, recombinant DH5αMCR cells were resuspended at 2×10^8/ml in PBS (pH 7.0) plus 2% D-mannose (Sigma) to inhibit mannose-sensitive adherence due to type 1 fimbriae before being subjected to a cell adherence assay at 37°C under a 10% CO_2 atmosphere (16).

To measure adherence inhibition with antibodies, *C. difficile* strain 79-685 was washed twice, heat shocked (60°C for 20 min) or not, and preincubated with antibodies (1/10, 1/100, and 1/500 dilutions in TNT [10 mM Tris-Cl {pH 8}, 150 mM NaCl, 0.05% Tween 20] plus 5% nonfat milk) for 30 min before being added to cells. When inhibitions were carried out with purified Cwp66-N and Cwp66-C recombinant proteins, Vero cells were preincubated for 15 min at 37°C under a 5% CO_2 atmosphere with 10 and 50 μg of protein per ml in Dubelcco's modified Eagle's medium (Eurobio, Paris, France) and then washed twice in PBS before bacteria were added to the cells for 1 h at 37°C under anaerobic conditions. The statistical differences between various conditions were assessed by Student's *t* test.

Computer analyses. Nucleotide and amino acid sequence alignments were performed with the ClustalW program (European Bioinformatics Institute, Cambridge, United Kingdom). Homology searches were conducted with Fasta3 (European Bioinformatics Institute) or Blast 2.0 (National Institute for Biotechnology Information, Washington, D.C.). RNAdraw was used to search for hairpins in RNA sequences (22).

Data imaging. Pictures were scanned with a Agfa Snapscan 1212 scanner, and the scanned images were printed with an Epson 700 printer.

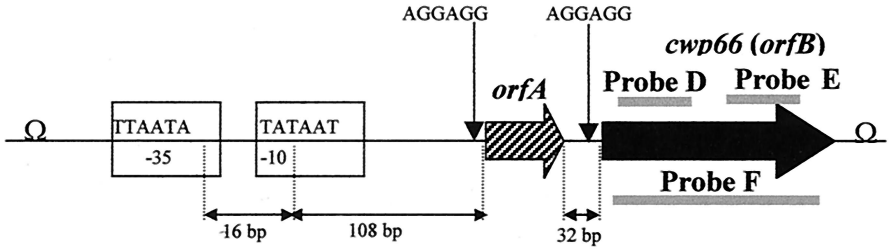
Nucleotide sequence accession number. The sequence of the 3.7-kb DNA fragment from strain 79-685, including ORFA and the *cwp66* gene, has been deposited in the GenBank database under accession no. AF093106. The sequence of the *cwp66* gene from strain C-253 has been deposited under accession no. AF194870.

RESULTS

Cloning of the gene encoding Cwp66. Our previous results have demonstrated that adherence of *C. difficile* 79-685 to cultured cells is increased by various stresses (34), and it is likely that the proteins mediating adherence are present in a significant amount on the bacterial surface after a heat shock (16). To identify these proteins, we screened a λZapII phage library (16) with antibodies prepared against heat-shocked bacteria in toto and adsorbed against non-heat-shocked *C. difficile*. Twenty-four positive plaques were obtained among 50,000 screened, and corresponding phages were converted into phagemids by in vivo excision. *E. coli* cells carrying these plasmids were not adherent to Vero cells (data not shown). After

FIG. 1. (A) Genetic organization of the 3.7-kb genomic fragment of *C. difficile* strain 79–685 carrying *orfA* and *cwp66*. Putative promoter consensus sequences (−10, −35) and ρ-independant terminator sequences (Ω) are shown. Two ribosome binding sites are indicated (RBS), as are the sizes and localizations of the three probes used in this study. (B) Alignment of the repeats of the Cwp66 protein, respectively, in the N-terminal (N-ter) and the C-terminal (C-ter) domains. The length of N-terminal repeats is between 52 and 60 amino acids, whereas the length of the C-terminal repeats is between 21 and 23 amino acids. The amino acid positions in the Cwp66 protein are indicated. ∗ and +, two and three identical amino acids, respectively; ◦, functionally identical amino acids (A, S, and T; D and E; N and Q; R and K; I, L, M, and V; F, Y, and W). (C) Alignment of amino acid sequences of Cwp66 of *C. difficile* strains 79-685, C-253, and CwlB of *B. subtilis* with the ClustalW program. Identical amino acids are shaded in gray.

A



B

N-ter

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033  LTGSGRWETAIKISQAGWTKSESAVLV.......NDNSIADALSATPFAKAKDAPILLT 85
132 RISGNSRYDTSLKLAEKLDREKSISKIVVVN....GEKGLADAVSVGAIAAQENMPIILS 137
230 RIAGSSRSETNAKIIEEFYKDTDIKNIYVTKDGTKNKNDLIDSLAVGVLAAKNSSPIILA 289
    ++ *++* *+  ++ +  °°°°°*° ***       *°* **+***** °+*   °++*+°
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C-ter

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375 ITVEFDGVF.KQSITIDMPNGDV 396
435 IENTSDGDI..WIITIDADAKDV 455
465 ISNNAPGVIIKNSGKIDLVNGNE 488
    +°* °*+** *°***++° ****
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C

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Cwp66 79-685  MKISKKIVSLLTMTFLTVTLYGNTSNASTK-DTLTGSGRWETAIKISQAGWTKSESAVLV
Cwp66 C-253   MNISKQIVSLLTMTFLTVTLYGNTSNASTK-DTLTGSGRWETAIKISQAGWTKSESAVLV
CwlB_Bacsu    ---MRSYIKVLTMCFLGLILFVPTALADNSVKRVGGSNRYGTAVQISKQMYSTASTAVIV

Cwp66 79-685  NDNSIADALSATPFAKAKDAPILLTQSNKLDSRTKAELKRLGVKNVYLIGGSIALSSEIE 120
Cwp66 C-253   NDNSIADALSATPFAKAKDAPILLTQSNKLDSRTKAELKRLGVKNVYLIGGSIALSSEIE
CwlB_Bacsu    GGSSYADAISAAPLAYQKNAPLLYTNSDKLSYETKTRLKEMQTKNVIIVGGTPAVSSNTA

Cwp66 79-685  KQLNAENINFERISGNSRYDTSLKLAEKLDREKSISKIVVVNGEKGLADAVSVGAIAAQE 180
Cwp66 C-253   KQLNAENINFERISGNSRYDTSLKLAEKLDREKSISKIVVVNGEKGLADAVSVGAIAAQE
CwlB_Bacsu    NQIKSLGISIKRIAGSNRYDTAARVAKAMG---ATSKAVILNGFL-YADAPAVIPYAAKN

Cwp66 79-685  NMPIILSDSENGTEVADNFIDSKDIAKSYVIGGTYSISSSVERSLPNATRIAGSSRSETN 240
Cwp66 C-253   NMPIILSDSENGTEVADNFIDSKDIAKSYVIGGTYSISNSVERSLPNATRIAGSSRSETN
CwlB_Bacsu    GYPILFTNKTSINSATTSVIKDKGISSTVVVGGTGSISNTVYNKLPSPTRISGSNRYELA

Cwp66 79-685  AKIIEEFYKDTDIKNIYVTKDGTKNKNDLIDSLAVGVLAAKNSSPIILA-GNKLDTTQKD 300
Cwp66 C-253   AKIIEEFYKDTDIKNIYVTKDGTKNKNDLIDSLAVGVLAAKNSSPIVLA-GNKLDTTQKD
CwlB_Bacsu    ANIVQKLNLSTS--TVYVS-----NGFSYPDSIAGATLAAKKKQSLILTNGENLSTGARK

Cwp66 79-685  VLNTKIIDKVTQIGGLGNEDAVKSIVDMQEKTKYTVETIEELNVAIKKADANDVIIFEPE 360
Cwp66 C-253   VLNTKIIDKVTQIGGLGNENVVEDILDIQEETKYTVETIDELNAAIKRADANDIIKFKPE
CwlB_Bacsu    IIGSKNMSNFMIIG---NTPAVSTKVANQLKNPVVGETIF-IDPGHGDQDSGAIGNGLLE

Cwp66 79-685  KDTNISDSFKIATNKAITVEFDGVFKQSITIDMPNGDVKNFGEISDDIRIDNIKKGTLIN 420
Cwp66 C-253   KEKTINNSFSIETKKTVTIELDGRYRQTITLDIPNGKFNNYAEIEGGVKLKNIKNESLVN
CwlB_Bacsu    KEVNLDIAKRVNTKLNASGALPVLSRSNDTFYSLQERVNKAASAQADLFLSIHANAN--D

Cwp66 79-685  EGSIQGIDIYSKNGCKIENTSDGDIWIITIDADAKDVYIENDGDITKISNNAPGVIIKNS 480
Cwp66 C-253   KGSIQDLDIYDENGCKIENESSGEIWFVTIVEEANDVYIVNSGDITKISNNSSSTIIRNS
CwlB_Bacsu    SSSPNGSETYYDTTYQAANSK-------RLAEQIQPKLAANLGTRDRGVKTAAFYVIKYS

Cwp66 79-685  GKIDLVNGNEQPAISGKKPTTNDTEYNDERARGLSVSTKPCSIPEKNRVRVTISSEPKSS 540
Cwp66 C-253   GNIDTVTGKKEPAISGNKPKVNDTEKETKAARGLNPRVEACSVPKKDYVMITIPNSPKDS
CwlB_Bacsu    KMPSVLVETAFITNASDASKLKQAVYKDKAAQAIHDGTVSYYR----------------

Cwp66 79-685  RYKIYYRVVEDKPSAMYVGEKISVRSWDLASKSDGSFVEKAKNGSYIEVVEINTSTNKVS 600
Cwp66 C-253   RYKIYYRVVYNKPYAMDVGDKINIGEWTVAPTDEEPFLEKAKNGCYVEAVEVNTSTKEVS
CwlB_Bacsu    ------------------------------------------------------------

Cwp66 79-685  RWGRSNVTDDGF 610
Cwp66 C-253   RWGRTNATDDGF
CwlB_Bacsu    ------------
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Profiles by Dot blot /Strains	Serogroup
A1 : D + , E + , F +	
93-54	A
4811	K
79-685	S3
5036	X
A2 : D + , E - , F +	
W1194; Kohn	A
C-253; 545; 94-1456; 95-1078; 96-602; 96-1578	C
90-111; 93-136; 93-226; 93-296	D
1470	F
VPI-10463; 2022; 95-938; 96-392; 96-1348; 96-1827	G
90-204; 93-369	H
94-416; 96-631	K
M1; 630	nd
A3 : D - , E - , F +	
1351	B
3232; 93-379	D
2149; 89-638	H
7322	I
94-1155	K

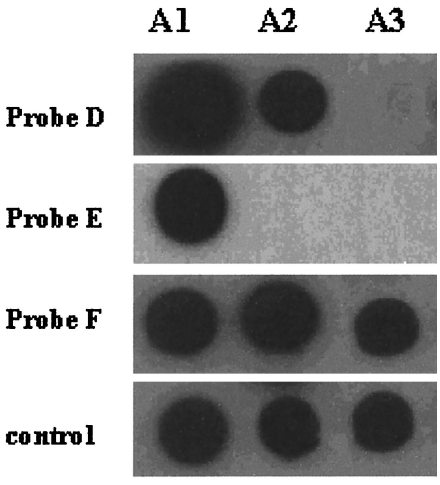

FIG. 2. Variability of the *cwp66* gene among *C. difficile* strains. Genomic DNAs from 36 strains of *C. difficile* belonging to various serogroups were hybridized with probe D, corresponding to the 5′ half of the gene; probe E, corresponding to the 3′ half of the gene; and probe F, corresponding to nearly the complete open reading frame. Hybridizations were performed under high-stringency conditions. nd, not determined.

sequencing of inserts, four were identical and were found to carry two successive open reading frames: (i) *orfA* (651 bp), encoding a 217-amino-acid, predominantly hydrophilic and charged protein (24.9 kDa), which displays no homology to known bacterial proteins; and (ii) *orfB*, showing homology to *cwl*B of *Bacillus subtilis*, which encodes an autolysin.

The two open reading frames are in an operon-like structure (Fig. 1A): they are separated only by 32 bp, and there is no prominent transcriptional terminator after *orfA*. Furthermore, the nucleotide sequence upstream from *orfA* carries a sequence resembling the σ^A binding site (consensus sequence, TTGACA-N_{17}-TATAAT) (12). *orfB* is likely to be transcribed from the promoter of *orfA* since no prominent promoter structure could be identified in the intergenic region. Upstream from this promoter and downstream from *orfB* there are hairpin loops with calculated free energies of −31.3 and −61.3 kJ/mol (37°C), respectively, which could represent transcriptional terminators. Inasmuch as *orfA* was unlikely to encode an exported or surface-associated protein, we focused our investigations on *orfB*.

Structure of the *cwp66* gene. The complete *orfB* of strain 79-685 is composed of 1,830 bp. The corresponding gene was named *cwp66* (for "clostridial wall protein 66 kDa"), and the corresponding protein was designated Cwp66. Southern hybridization with the *cwp66*-specific probe of total DNA of strain 79-685 digested with six enzymes showed that only one copy of the gene was present (data not shown).

Cwp66 is a 610-amino-acid protein with a calculated molecular mass of 66,323 Da and a pI of 5.3. Examination of the primary and secondary structures of Cwp66 reveals several features of bacterial surface proteins: (i) the protein is rich in hydrophilic (54%) and charged (27%) residues; (ii) the protein has a characteristic 27-amino-acid leader peptide, which has three charged lysines in the first six residues and is followed by a hydrophobic core with a probable signal cleavage site between Ala-27 and Ser-28 (33), suggesting that Cwp66 is exported to the membrane or secreted; (iii) the protein has a domain structure defined by sequence homologies and secondary structure (prediction obtained with SOPM [11]). The 283-amino-acid N-terminal domain (residues 29 to 312) shows 35% identity and 56% similarity to *N*-acetylmuramoyl-L-alanine amidase CwlB of *Bacillus subtilis* (GenBank accession no. Q02114) and *N*-acetylmuramoyl-L-alanine amidase modifier precursor CwbA of *B. subtilis* (Q02113), and 32% identity to a 62-residue fragment of *Listeria monocytogenes* internalin B (AF121040). The 289-amino-acid C-terminal portion shows remote homology (20 to 30% identity) to cell wall-associated proteins such as a cell surface antigen of *Rickettsia prowazekii* (AJ235273) and a surface-exposed protein of *Rickettsia typhi* (P96989). The domain structure is also conspicuous at the level

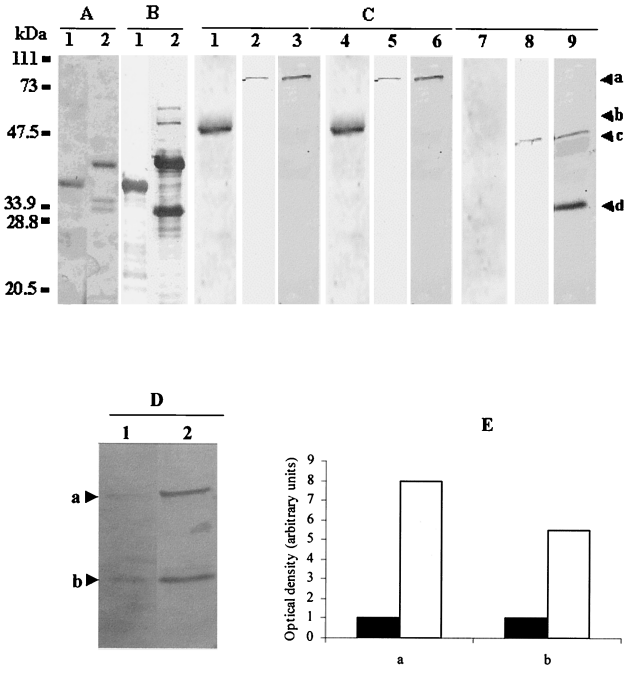

FIG. 3. Cwp66 protein purification and surface localization. (A) Purification of the *C. difficile* Cwp66 N-terminal domain (Cwp66-N) and C-terminal domain (Cwp66-C) overexpressed in *E. coli* BL21 SDS-PAGE gels are shown. Final eluates after cleavage of the GST part, showing the presence of a 35-kDa protein corresponding to Cwp66-N (lane 1) and a 44-kDa protein corresponding to Cwp66-C (lane 2), are shown. (B) Immunoblot analysis of *C. difficile* Cwp66-N (lane 1) and Cwp66-C (lane 2). After SDS-PAGE, the purified protein was transferred to a membrane and incubated with the corresponding antibodies (dilution, 1/2,000). (C) Immunoblot analysis of cytoplasmic (lanes 1 to 3), membrane (lanes 4 to 6), and cell wall (lanes 7 to 9) protein extracts of *C. difficile* 79–685. Equivalent amounts of proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with anti-PepC antibody as a control for cytoplasmic and membrane fractions (lanes 1, 4, and 7), anti-Cwp66-N (lanes 2, 5, and 8), and anti-Cwp66-C (lanes 3, 6, and 9). Antibodies were used at 1/2,000 dilution. (D) Immunoblot analysis of cell wall protein extract of non-heat-shocked (lane 1) and heat-shocked (lane 2) *C. difficile* 79– 685 revealed with the Cwp66-C antiserum. (E) Relative levels of Cwp66 before and after heat shock, determined by densitometry scanning of the immunoblot (D). The optical density of bands corresponding to 50-kDa (lane 1) and 30-kDa (lane 2) protein fragments before (■) and after (□) heat shock is shown.

of the secondary structure: the N-terminal half of the protein is characterized by a mostly α-helical conformation, whereas the C-terminal domain is predicted to exhibit a predominantly extended-strand formation. The N-and C-terminal domains carry three imperfect intramolecular repeated sequences (Fig. 1B).

Interstrain variability of the *cwp66* gene. To investigate the conservation of the *cwp66* gene region in different strains, 36 strains of *C. difficile* including 10 reference strains and 26 hospital isolates (Table 1) were tested for the presence of the *cwp66* gene by dot blot hybridization, using three different probes: probe D, corresponding to the 5′ part of the gene; probe E, corresponding to the 3′ part of the gene; and probe F, which covers 77% of the gene (Fig. 1A). All the strains examined hybridized with probe F (Fig. 2). Three groups of strains could be defined: (i) strains which hybridized with all three probes, such as our reference strain 79-685 (group A1); (ii) strains which were recognized by probe D and F, thus having variations in the C-terminal domain (group A2); and (iii) strains which hybridized with probe F but not with probe D or E (group A3).

The gene from another virulent strain (C-253) was isolated by screening a genomic library constructed in λZap Express with probe F and sequenced. A comparison of the deduced amino acid sequences of Cwp66 of the two strains and CwlB of *B. subtilis* is shown in Fig. 1C. During the latter part of this project, the genome sequence of another virulent *C. difficile* strain, 630, became available on the Internet (www.sanger.ac.uk). The Cwp66 proteins of strains C-253 and 630 are nearly identical (99.7% identity) and also exhibit a domain structure. The N-terminal portion of the proteins is well conserved between 79-685 and the two other strains (99.3% identity), whereas the C-terminal domain is more variable (58% identity).

Expression, purification, immunological detection, and surface localization of Cwp66. No recombinant protein in *E. coli* was recovered when the entire *cwp66* gene, amplified by PCR and cloned into the *E. coli* expression vector pGEX-6P-1, was used. This protein could be toxic for *E. coli*. Subsequently, the two domains of the *cwp66* gene (the 5′ region from bases 104 to 941 and the 3′ region from bases 929 to 1830) were expressed and purified separately.

The GST–Cwp66-N and GST–Cwp66-C fusion proteins were purified from IPTG-induced bacterial lysates by affinity chromatography on glutathione-Sepharose, and the GST part of the proteins was cleaved off with Prescission protease. As shown in Fig. 3A, a major 35-Da band and a 44-kDa band corresponding to Cwp66-N (residues 35 to 312) and Cwp66-C (residues 313 to 610), respectively, were observed in the final eluate by SDS-PAGE. These two bands were cut out from the gel and injected into rabbits in order to obtain polyclonal antibodies. Anti-Cwp66-N antibodies reacted in immunoblots with the purified 35-kDa Cwp66-N protein (Fig. 3B, lane 1) in *C. difficile* cytoplasmic and membrane extracts with an 80-kDa protein (Fig. 3C, lanes 2 and 5) and in *C. difficile* cell wall extract with a 50-kDa protein (lane 8). Anti-Cwp66-C antibodies recognized the same 80- and 50-kDa proteins in *C. difficile* cytoplasmic, membrane, and cell wall extracts, respectively (lanes 3, 6, and 9) and, in addition, a 30-kDa protein in the cell wall extract (lane 9). The latter protein is also present in the final purification eluate of Cwp66-C (Fig. 3B, lane 2) and is likely to represent a cleavage product of the purified 44-kDa Cwp66-C. It should be noted that purification was carried out in the absence of protease inhibitors. Cwp66 was not detected in the supernatant extract (data not shown).

As shown in Fig. 3D and E, in an immunoblot revealed by anti-Cwp66 C antibodies, the 50- and 30-kDa protein fragments appeared overexpressed in surface extracts after a heat shock compared with at 37°C.

To assess if Cwp66 is surface exposed, anti-Cwp66-N and anti-Cwp66-C antibodies were used as primary antibodies for immunogold labeling of the N- and C-terminal portions of the protein. As shown in Fig. 4A and B, with anti-Cwp66-N antibodies, few gold particles were present on the cell surface of non-heat-shocked and heat-shocked bacteria. Furthermore, in

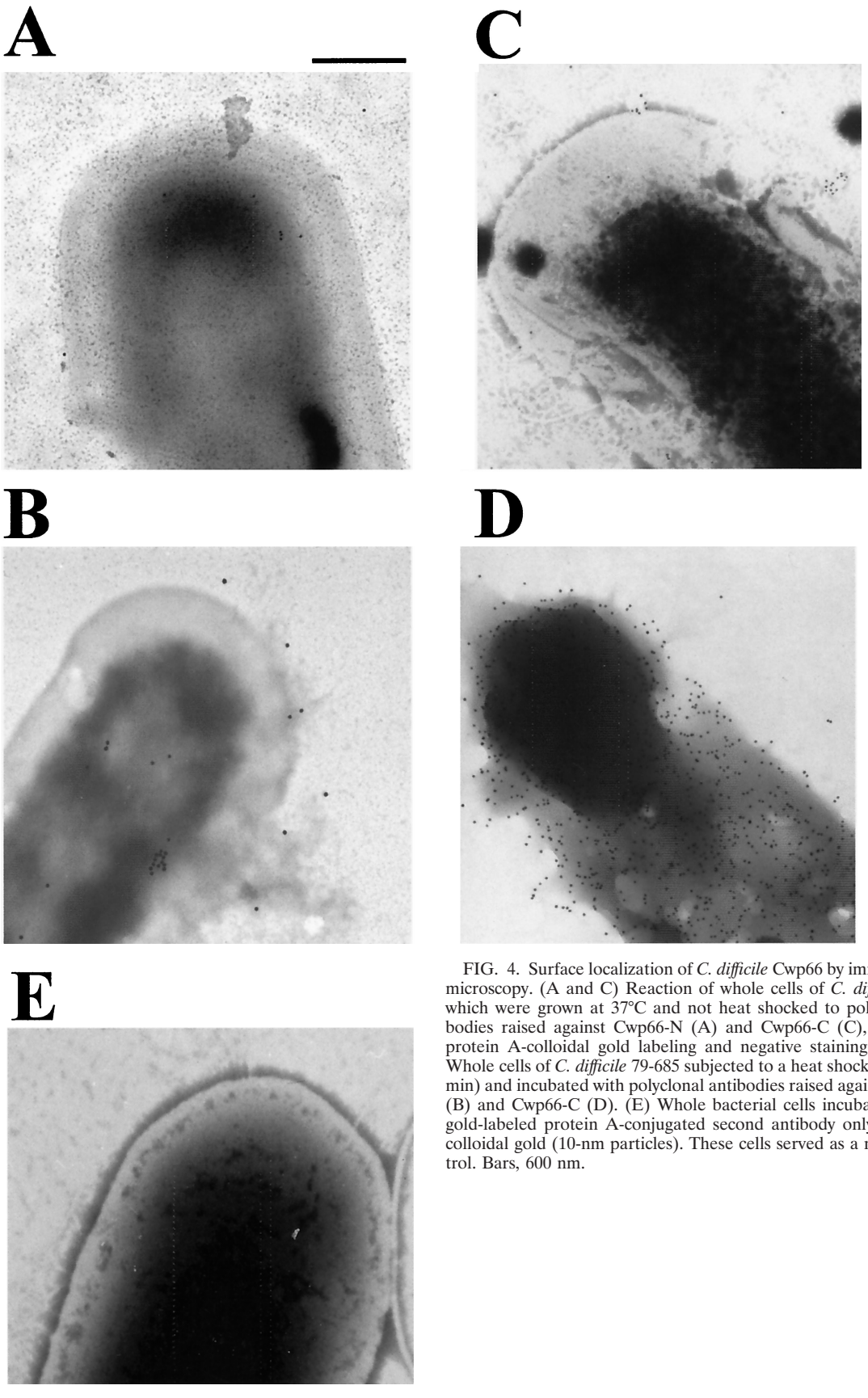

FIG. 4. Surface localization of *C. difficile* Cwp66 by immunoelecton microscopy. (A and C) Reaction of whole cells of *C. difficile* 79–685 which were grown at 37°C and not heat shocked to polyclonal antibodies raised against Cwp66-N (A) and Cwp66-C (C), followed by protein A-colloidal gold labeling and negative staining. (B and D) Whole cells of *C. difficile* 79-685 subjected to a heat shock (60°C for 20 min) and incubated with polyclonal antibodies raised against Cwp66-N (B) and Cwp66-C (D). (E) Whole bacterial cells incubated with the gold-labeled protein A-conjugated second antibody only, containing colloidal gold (10-nm particles). These cells served as a negative control. Bars, 600 nm.

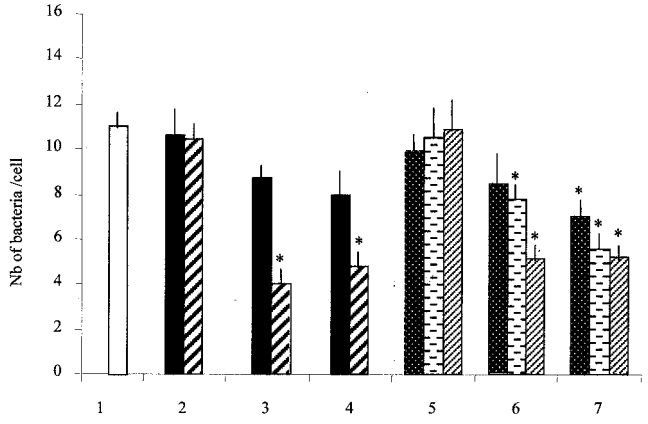

FIG. 5. Inhibition of heat-shocked *C. difficile* adherence to Vero cells by antibodies and purified Cwp66 domains. Adherence was determined in the presence of PBS (bar 1), BSA (negative control; a nonadhesive protein) (bars 2), purified Cwp66-N (bars 3), purified Cwp66-C (bars 4), anti-FliC (antibodies against *C. difficile* flagellin as a negative control; FliC is a nonadhesive protein) (bars 5), anti-Cwp66-N (bars 6), and anti-Cwp66-C (bars 7). Proteins were used at 10 μg/ml (■) and 50 μg/ml (▨); antibodies were tested at 1/10 (▩), 1/100 (▤), and 1/500 (▨) dilutions. Values are the means and standard deviations of at least four separate experiments. *, statistically significant difference (Student's *t* test; $P < 0.05$) compared with controls.

the non-heat-shocked bacteria (Fig. 4C), little immunolabeling of bacteria was observed with anti-Cwp66-C antibodies. In contrast, with anti-Cwp66-C antibodies, gold beads were observed as a uniform coating on the cell surface of heat-shocked bacteria, indicating that Cwp66 is evenly distributed and that its C-terminal domain is surface exposed (Fig. 4D). A control incubated in the absence of antibodies to Cwp66 did not reveal any gold particles on the cell surface (Fig. 4E).

Role of Cwp66 in cell attachment. Surface localization of Cwp66 suggests that it could play a role in adherence to cells. The role of Cwp66 in adherence to Vero cells was investigated by using polyclonal antibodies to Cwp66-N and to Cwp66-C as competitive inhibitors. As shown in Fig. 5, coincubation of heat-shocked bacteria with anti-Cwp66-N and with anti-Cwp66-C at a dilution of 1/10 demonstrated a relative adherence of 85 and 70%, respectively, compared with control adherence of 100% (adherence with the anti-FliC serum at the same dilution) and coincubation with the antibodies at a dilution of 1/100 demonstrated a relative adherence of 70 and 51%, respectively. The adherence level of heat-shocked *C. difficile* incubated with antibodies used at a dilution of 1/500 were 47 and 48% with anti-Cwp66-N and anti-Cwp66-C, respectively. If the bacteria were not heat shocked, the antibodies did not inhibit adherence (data not shown). Furthermore, competitive inhibition assays using purified Cwp66-N and Cwp66-C proteins confirmed these results (Fig. 5): the two purified proteins did not significantly reduce adherence when used at 10 μg/ml but inhibited cell adherence by 60 and 54%, respectively, when used at a concentration of 50 μg/ml. In contrast, no inhibition was observed when competitive inhibition was carried out with the nonadhesive protein BSA.

DISCUSSION

Our laboratory has been attempting to identify the factors involved in colonization of the intestine by *C. difficile* (8, 16, 34). The Cwp66 protein described in this communication was identified by immunological screening of a genomic library with antibodies raised against surface proteins of heat-shocked bacteria. This suggests that Cwp66 could be a surface-associated heat shock protein.

The amino acid sequence of Cwp66 indicated the presence of two domains, suggesting that this protein, like a number of surface proteins of gram-positive bacteria, many of which are adhesins, may be multifunctional. Because of the homology of the N-terminal domain of Cwp66 to the peptidoglycan recognition domain in the CwlB autolysin of *B. subtilis*, which after secretion is involved in attachment of the protein to the bacterial cell wall (20), it is likely that this domain could play the same role in Cwp66. This hypothesis is further supported by the facts that the protein does not appear to have transmembrane-spanning regions and that Cwp66 does not possess the cell wall-anchoring motif LPXTG, which has been found in numerous adhesins of gram-positive bacteria but never in clostridia (23). There are several examples in the literature of secreted proteins which bind the cell wall and are implicated in adhesion and invasion. This is the case for several proteins with autolytic properties such as P60 of *L. monocytogenes* (19, 36), Aas of *Staphylococcus saprophyticus* (14), and AtlE of *S. epidermidis* (13). There are also some examples of proteins displaying homology to autolysins which nevertheless do not express this activity, such as the invasion protein InlB of *L. monocytogenes* (4) and the surface protein PspA of *Streptococcus pneumoniae* (37). The adhesive properties of CwlB have not been investigated. Since *C. difficile* exhibits autolytic activities (data not shown), we cannot rule out an autolytic activity for Cwp66, but unfortunately we were not able to test this activity since we were not able to express and purify the protein in its entirety. The C-terminal portion of Cwp66 shows remote homology to bacterial cell surface-associated proteins, further supporting the hypothesis that this part of the protein could be surface exposed. By immunoelectron microscopy, it is evident that the C-terminal domain is exposed to the outside after heat shock whereas the N-terminal part may be embedded in the cell wall, inaccessible to the antibodies.

Finally, the presence of Cwp66 in surface extracts confirms that this protein is cell wall associated. Both anti-Cwp66-N and anti-Cwp66-C antibodies recognized a 50-kDa protein in cell wall extracts of *C. difficile* and an 80-kDa protein in cytoplasmic and membrane extracts. Many surface proteins of gram-positive bacteria can undergo posttranslational modifications or may have features that cause slow migration in SDS-polyacrylamide gels (9, 27), and the 80-kDa protein probably represents retarded migration of Cwp66. The 50- and 30-kDa bands in cell wall extracts are probably the result of a specific proteolytic cleavage in the C-terminal part of the protein, and it is conceivable that heat shock induces this cleavage. We are also investigating the role of heat shock in the transcription of the *cwp66* gene.

Surface localization and the presence of repeat motifs in Cwp66 suggested that this protein may exhibit adhesive activity. The two antisera partially inhibited adherence to tissue

culture cells, confirming that Cwp66 is an adhesin. Competitive inhibitions with the purified protein validated this result. Antibodies raised against the N-terminal domain could prevent fixation of the secreted Cwp66 to the cell wall and thereby indirectly impede adherence. We believe that the motifs responsible for cell attachment reside in the surface-exposed C-terminal part of the protein, although it is possible for adhesins of gram-positive bacteria to contain adhesive motifs in two domains, as is the case for proteins M and F of *Streptococcus pyogenes* (24, 25, 35). The fact that only partial inhibition was observed with the antibodies suggests that several adhesins may play a role in cell adherence of *C. difficile*. The fact that adherence of non-heat-shocked bacteria was not inhibited by antibodies corroborates the specificity of inhibition and suggests that heat shock could induce a conformational change or cleavage in the protein, which then gains adhesive properties.

We studied the variability of the *cwp66* gene among 36 strains of *C. difficile* belonging to 11 different serogroups (7) in order to establish virulence profiles. All strains carry the *cwp66* gene. We were able to classify the strains into three groups, which, however, did not correlate with toxinogenicity or serogroups, since strains from serogroups A, K, and D can be found in two or three different groups. Sequence analysis of Cwp66 from three virulent *C. difficile* strains, 79-685, C-250, and 630, confirmed the pronounced variations of the 3′ part of the gene. This result upholds the hypothesis that the C-terminal domain is surface exposed and thereby is subjected to immune selection.

In conclusion, we report here the characterization of a surface protein of *C. difficile* with adhesive properties. The isolation of Cwp66 is an important step in the characterization of the colonization process by *C. difficile*. In fact, to our knowledge, Cwp66 is the first adhesin ever identified in clostridia. Like other bacteria, *C. difficile* may possess multiple adhesins, and our aim is to elucidate the role of each. Furthermore, since adhesins are attractive targets for the development of vaccines, investigations are under way in our laboratory to evaluate the capacity of Cwp66 to trigger a protective immune response.

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